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Biochimica et Biophysica Acta 1452 (1999) 263–274

BIOCHIMICA ET BIOPHYSICA ACTA

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Possible involvement of cytochrome *c* release and sequential activation of caspases in ceramide-induced apoptosis in SK-N-MC cells

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Received 18 March 1999; received in revised form 23 August 1999

Abstract

Ceramide is characterized as a second messenger of apoptosis induced by various agents such as tumor necrosis factor (TNF-α), Fas ligand, hydrogen peroxide, heat shock and ionizing radiation. In this study, we investigated the mechanism of ceramide-induced apoptosis using a human neuroblastoma cell line, SK-N-MC. *N*-Acetyl-sphingosine (C2-ceramide), a cell-permeable ceramide analogue, was able to induce apoptosis in SK-N-MC cells as estimated by DNA fragmentation and chromatin condensation. C2-ceramide-induced DNA fragmentation was blocked by caspase inhibitor (Z-Asp-CH_2-DCB). An increase in caspase-3 (CPP32)-like protease activity was evident during C2-ceramide-induced apoptosis, suggesting that caspases are involved in this apoptosis. Moreover, enzymatic cleavage of VDVAD-AFC and LEHD-AFC (specific substrates for caspase-2 and -9, respectively) was increased by treatment with C2-ceramide. To elucidate which types of caspase are activated in C2-ceramide-treated cells, we performed Western blot analysis using antibodies against each isoform. Both proforms of caspase-2 and -3 were decreased in response to C2-ceramide in a time-dependent manner. Mitochondrial cytochrome *c* is also time-dependently released into the cytosol in response to treatment with C2-ceramide. Addition of cytochrome *c* into the S-100 fractions prepared from SK-N-MC cells could activate caspase-2 in cell-free systems. These results suggest the possibility that cytochrome *c* released to the cytosol can activate caspases (caspase-9, -3, and -2) during C2-ceramide-induced apoptosis of SK-N-MC cells.

Keywords: Ceramide; Neuron; Apoptosis; Caspase; Cytochrome *c*

1. Introduction

Sphingolipids have been known to be structural lipids that are constituents of the plasma membrane; they are also, however, widely recognized as intracellular second messengers. Signals via cell-surface receptors such as tumor necrosis factor (TNF) or Fas (CD95/Apo1), and various stress stimuli such as X-rays, hydrogen peroxide, heat shock, or ultraviolet-B irradiation activate sphingomyelinase (SMase) and then generate ceramide through the hydrolysis of sphingomyelin. The production results in cell death by means of apoptosis [1–3]. Ceramide is therefore considered to be an apoptotic second messenger, since synthetic cell-permeable ceramide can also induce apoptosis in hematocytes [4] and neuronal cells [5,6]. In contrast, cell-permeable ceramide has an anti-apoptotic effect to protect the cells against glutamate or amyloid β-peptide toxicity and nerve growth factor deprivation [7,8]. These results suggest that different signal transductions exist

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0167-4889/99/$ – see front matter
PII: S0167-4889(99)00131-7

downstream from ceramide production in each neuronal cell type.

Genetics studies in *Caenorhabditis elegans* have identified the ced-3 gene, which is required for all developmentally programmed cell deaths [9]. First, the cysteine protease interleukin-1β converting enzyme (ICE) was identified as the mammalian Ced-3 homologue [10]. Subsequently, 13 additional mammalian Ced-3-related cysteine proteases have been isolated and termed as caspases that play a central role in the execution of apoptosis. All caspase family genes encode proenzyme forms that require proteolytic cleavage for activation. From an analysis of the caspase-1 (ICE)- and caspase-3 (CPP32)-deficient mouse, each caspase has been shown to play a different role in different cells, tissues, or apoptotic stimuli [11,12]. Nedd-2, murine caspase-2 (Ich-1), has been isolated and shown to be highly expressed during early embryonic brain development but not in the adult brain [13,14]. A decrease in caspase-2 levels in response to antisense technology delays apoptosis induced by trophic factor deprivation in neuronal cell line, PC12 cells [15]. The involvement of caspase-2 in other stimulus-induced neuronal apoptosis compared to that of caspase-3 in apoptosis by multiple apoptotic stimuli such as TNF-α, Fas, granzyme B, and ceramide, however, remains unknown [16–22].

In the current study, we therefore investigated the involvement of caspases, particular caspase-9 and caspase-3-like proteases, in ceramide-induced apoptosis in a human neuroblastoma cell line, SK-N-MC.

2. Materials and methods

2.1. Materials

SK-N-MC cells were obtained from the American Type Culture Collection (USA). *N*-Acetyl-sphingosine (C2-ceramide), sphingosine, and sphingomyelinase (from *Staphylococcus aureus*) were purchased from Sigma (USA). The cytotoxicity Detection Kit (lactate dehydrogenase (LDH)) and high fidelity PCR system were obtained from Boehringer Mannheim (UK). Benzyloxycarbonyl-Asp-CH_2OC(O)-2, 6-dichlorobenzene (Z-Asp-CH_2-DCB), *N*-acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide (Ac-DEVD-MCA), and *N*-acetyl-Tyr-Val-Ala-Asp-4-methylcoumaryl-7-amide (Ac-YVAD-MCA) were from Peptide Institute (Japan). Caspase-2 and caspase-9 fluorometric protease assay kits were from MBL (Japan). Oligo$(dT)_{12-18}$ primer and superscript reverse transcriptase (RT) were obtained from Gibco BRL (USA). Anti-ICE and CED-3 homologue 1 (anti-Ich-1; caspase-2) antibody, anti-cysteine protease p32 (anti-CPP32; caspase-3) antibody, anti-α-tubulin antibody, and anti-poly(ADP-ribose) polymerase (anti-PARP) antibody (clone C-2-10) were from Santa Cruz (USA), Transduction Laboratories (USA), Seikagaku (Japan) and Clontech (USA), respectively. Anti-caspase-6, -7, -8, and -10 antibodies and anti-cytochrome *c* antibodies (7H8.2C12 and 6H2.B4) were from Pharmingen (USA). The TNT T7 Coupled Reticulocyte Lysate System was purchased from Promega (USA).

2.2. Cell culture

SK-N-MC cells were maintained in minimum essential medium supplemented with 10% fetal calf serum (FCS) in a humidified incubator containing 5% CO_2.

2.3. LDH leakage assay

Cell death was estimated by the LDH leakage method using a Cytotoxicity Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. Cells were seeded at 1.5×10^5/well in triplicate in 12-well plates for 48 h prior to treatment with C2-ceramide (0–20 μM) for 24 h in serum-free medium. LDH activity was measured as optimal density at 492 nm, and LDH leakage (%) was defined as the ratio of LDH activity in the culture medium to the total activity (% = (extracellular activity)/(extracellular activity+remaining cellular activity)).

2.4. Apoptosis assay

For the DNA fragmentation assay, the cells were lysed in a lysis buffer (10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% Triton X-100) and incubated for 20 min at 4°C. The samples were centrifuged at $27\,000\times g$ for 15 min at 4°C. The supernatants were incubated with 40 μg/ml proteinase K for 30 min at 37°C and extracted with an equal volume of phenol,

phenol/chloroform (v/v; 1:1), and chloroform. The DNA was precipitated from the supernatants with a 1/10 volume of 3 M sodium acetate and 2.5 vols. of ethanol and then treated with 40 μg/ml RNase A for 1 h at 37°C. The recovered DNA was then analyzed by electrophoresis on a 1.8% agarose gel and visualized with ethidium bromide. For fluorescent nuclei staining, the cells were harvested and fixed for 20 min in PBS containing 1% glutaraldehyde. After fixation, the cells were washed four times with PBS and then stained with 1 mM Hoechst 33258, and the nuclei were visualized by fluorescent microscopy as described in [23].

2.5. Measurement of caspase activity

The cells were collected, washed with PBS, and suspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. After the addition of 10 μM digitonin, the cells were incubated at 37°C for 10 min and then centrifuged at 15 000 rpm for 20 min. The clear lysate (50 μg of protein) was incubated at 37°C with 50 μM DEVD-MCA for 30 min or 50 μM YVAD-MCA for 1 h. The amounts of released 7-amino-4-methylcoumarin (AMC) were measured with a fluorescence spectrophotometer (Hitachi F-2000) with excitation at 380 nm and emission at 460 nm. Caspase-2 and -9 activities were measured using caspase-2 and caspase-9 fluorometric protease assay kits. The assay for caspase-2 or caspase-9 activity is based on detection of cleavage of substrate VDVAD-7-amino-4-trifluoromethylcoumarin (AFC) or LEHD-AFC. The amounts of released AFC were measured with a fluorescence spectrophotometer (Molecular Devices) with excitation at 400 nm and emission at 505 nm.

2.6. RT-PCR analysis

Total RNA was prepared from SK-N-MC cells and Jurkat cells using the acid guanidium thiocyanate-phenol-chloroform extraction method. Total RNA (2 μg) was reverse-transcribed in a mixture containing oligo(dT)$_{12-18}$ primer (Gibco BRL) and superscript reverse transcriptase (Gibco BRL). PCR was carried out in a 20 μl reaction volume containing 2 μl of cDNA mixture, 1×Expand high fidelity buffer containing 1.5 mM $MgCl_2$, 200 μM each dNTP, 200 μM each primer, and 0.7 U of high fidelity PCR system enzyme mixture (Boehringer Mannheim) as described in [24]. The DNA was denatured for 2 min at 94°C prior to each PCR cycle (17–40 cycles) of 94°C for 1 min, each annealing temperature (50–62°C) for 1 min, 72°C for 1 min, followed by 4 min at 72°C before refrigeration. The primers employed are listed here: caspase-1 (upstream) 5′-AAC CCA GCT ATG CCC ACA TCC-3′; caspase-1 (downstream) 5′-TTA ATG TCC TGG GAA GAG GTA-3′; caspase-2 (upstream) 5′-GTT ACC TGC ACA CCG AGT CAC G-3′; caspase-2 (downstream) 5′-GCG TGG TTC TTT CCA TCT TGT TGG TCA-3′; caspase-3 (upstream) 5′-GAA TAT CCC TGG ACA ACA-3′; caspase-3 (downstream) 5′-ACG CCA TGT CAT CAT CAA-3′; caspase-4 (upstream) 5′-GGT CAT CAT TGT CCA GGC-3′; caspase-4 (downstream) 5′-CCA TTG TGC TGT CTC TCC-3′; caspase-5 (upstream) 5′-ACC ACA TGC TAA AGA ACA-3′; caspase-5 (downstream) 5′-CGA TTT GCA GAA GAG GTT-3′; caspase-6 (upstream) 5′-ACC CGC AGG TTT TCA GA-3′; caspase-6 (downstream) 5′-CAT GAG CCG TTC ACA GT-3′; caspase-7 (upstream) 5′-AGC CTG GGT TTT GAC GTG-3′; caspase-7 (downstream) 5′-ACC GTG GAA TAG GCG AAG-3′; caspase-8 (upstream) 5′-GGA CTG CTT CAT CTG CTG-3′; caspase-8 (downstream) 5′-ATC TGT TTC CCC ATG TTT-3′; caspase-9 (upstream) 5′-TAA CAG GCA AGC AGG AAA-3′; caspase-9 (downstream) 5′-TCT TGG CAG TCA GGT CGC-3′; caspase-10 (upstream) 5′-AGA AGT CCA GCT CAG CCT-3′; caspase-10 (downstream) 5′-ACT CGG CTT CCT TGT CTA-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (upstream) 5′-AAA CCC ATC ACC ATC TTC CAG-3′; and GAPDH (downstream) 5′-AGG GGC CAT CCA CAG TT TCT-3′. The number of cycles selected for each primer pair was found to produce a linear relationship between the input RNA and the resulting PCR products. The PCR products were analyzed on a 6.5% non-denaturing polyacrylamide gel.

2.7. Western blot analysis

The cells were collected and suspended in 50 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leu-

peptin. The cells were then sonicated and centrifuged at 15 000 rpm for 20 min at 4°C. The supernatants (cytosol fractions) were used for the immunoblotting of ICH-1L (caspase-2), CPP32 (caspase-3), and α-tubulin and the pellet (membrane fractions) was used for the immunoblotting of poly(ADP-ribose) polymerase (PARP). For the immunoblotting of cytochrome *c*, the cells were collected and suspended in 10 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, and 250 mM sucrose and frozen in liquid nitrogen. After thawing on ice, the cells were disrupted by douncing 10 times using a dounce homogenizer (Wheaton), and the cells were then centrifuged at $2000 \times g$ for 5 min at 4°C. The supernatant was further centrifuged at $20\,000 \times g$ for 20 min at 4°C, and the cytosol fractions were used for immunoblotting. The indicated amounts of protein were separated on 8–15% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. The following antibodies were used as primary antibody: anti-human ICH-1L polyclonal goat antibody (Santa Cruz), anti-human CPP32 monoclonal antibody (Transduction Laboratories), anti-caspase-6, -7, -8, and -10, anti-human cytochrome *c* monoclonal antibody (7H8.2C12) (Pharmingen), anti-α-tubulin monoclonal antibody (Seikagaku), and anti-human PARP monoclonal antibody (Clontech). Either horseradish peroxidase conjugated anti-mouse IgG (Amersham) or horseradish peroxidase conjugated anti-goat IgG (Santa Cruz) was used as the secondary antibody. Detection of the bands was performed using the ECL system (Amersham).

2.8. Preparation of S-100 fractions from SK-N-MC cells

The cells were collected and suspended in buffer A (20 mM HEPES-NaOH (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml aprotinin). After incubation on ice for 15 min, the cells were homogenized and centrifuged at $2000 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $20\,000 \times g$ for 20 min at 4°C and further centrifuged at $100\,000 \times g$ for 1 h at 4°C. The resulting supernatant (S-100 fraction) was stored at −80°C and used for the in vitro cleavage assay. The immunodepletion of cytochrome *c* from SK-N-MC S-100 was carried out using an anti-cytochrome *c* antibody (6H2.B4) that recognized the native form of cytochrome *c*. 40 μl of this antibody were incubated with a 1:1 mixture of 50 μl of protein A and protein G-Sepharose beads (50% slurry) at 4°C for 3 h. The beads were collected and incubated with S-100 fractions for 12 h in a rotator at 4°C. The beads were subsequently pelleted by centrifugation at $10\,000 \times g$ for 15 min at 4°C. The supernatant was used as cytochrome *c*-depleted S-100.

2.9. In vitro cleavage assay

A cDNA encoding the full length of caspase-2 was cloned into a pCR3.1 vector (Invitrogen). An in vitro translation of caspase-2 was carried out using a TNT T7 transcription/translation kit (Promega) in the presence of [^{35}S]methionine according to the instructions of the manufacturer. A 1 μl aliquot of the in vitro-translated caspase-2 was incubated with 50 μg of the above in the indicated fractions, 1 mM dATP and 1 mM additional $MgCl_2$ at 30°C for 2 h in a final volume of 20 μl of buffer A. At the end of the incubation, 5 μl of 5×SDS buffer was added to each reaction. After boiling for 5 min, each sample was subjected to a 15% SDS-polyacrylamide gel and visualized by Fujix BAS 2000 (Fuji).

3. Results

3.1. Induction of apoptosis by ceramide

To investigate whether C2-ceramide induces the loss of viability in SK-N-MC cells, we estimated the LDH leakage as an index of cell death. Treatment with C2-ceramide induced LDH leakage in a dose-dependent manner (Fig. 1A). Furthermore, the treatment also resulted in internucleosomal DNA fragmentation (Fig. 1B) and chromatin condensation (Fig. 1C), both of which are biochemically and morphologically characteristic of apoptosis, suggesting the cell death by C2-ceramide is apoptosis. Treatment with 20 μM sphingosine, a sphingolipid breakdown product, also caused internucleosomal DNA fragmentation and morphological changes characteristic of apoptotic cells (Fig. 1B,C). To investigate the specificity of C2-ceramide-induced phenomena on

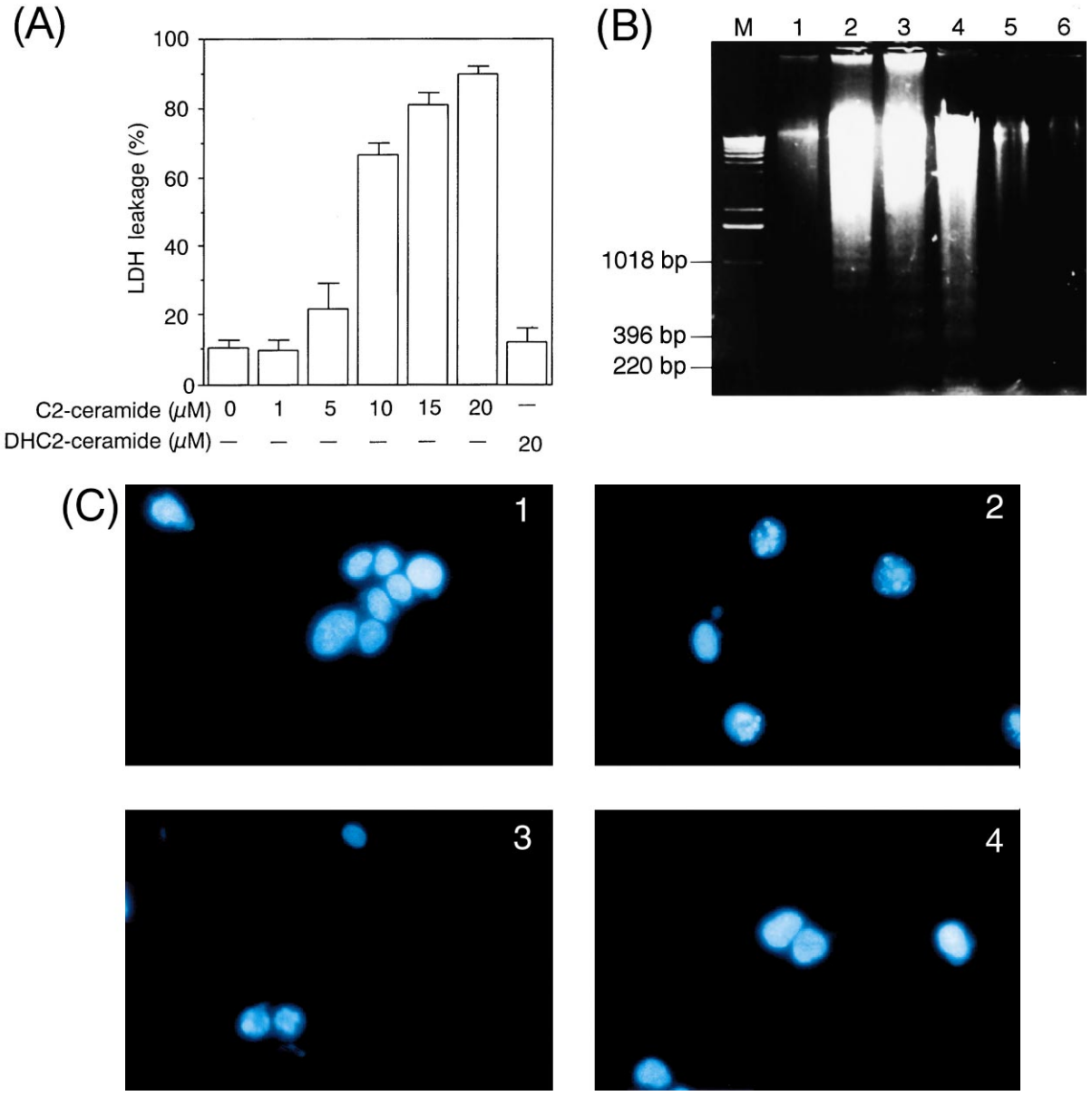

Fig. 1. Induction of apoptosis by C2-ceramide in SK-N-MC cells. (A) LDH leakage caused by C2-ceramide and C2-dihydroceramide (DHC2-ceramide). The cells were treated with the indicated concentrations (0–20 μM) of C2-ceramide or 20 μM DHC2-ceramide for 24 h and then subjected to an LDH leakage assay. Each value is the mean ± S.E. of three samples. (B) DNA fragmentation by treatment with ethanol vehicle (lane 1), 20 μM C2-ceramide (lane 2), 20 μM sphingosine (lane 3), 400 mU/ml SMase (lane 4), 20 μM DHC2-ceramide (lane 5), and 20 μM sphingosine-1-P (lane 6) for 6 h. (C) Nuclear staining of cells with the fluorescent dye Hoechst 33258 after treatment with ethanol vehicle (panel 1), 20 μM C2-ceramide (panel 2), 20 μM sphingosine (panel 3), and 20 μM DHC2-ceramide (panel 4) for 6 h.

apoptosis, we tested the effect of a structural analogue of ceramide. A ceramide analogue C2-dihydroceramide (DHC2-ceramide), which lacks the C4-5 *trans* double bond in the sphingolipid backbone necessary for the biological effects of ceramide [25], induces neither LDH leakage (Fig. 1A) nor characteristic changes in apoptosis (Fig. 1B,C). Furthermore, the addition of 20 μM sphingosine-1 phosphate, a metabolic product of sphingosine, also did not induce DNA fragmentation (Fig. 1B). On the other

hand, sphingomyelinase (SMase) catalyzes the hydrolysis of sphingomyelin, yielding ceramide. It has been reported that treatment with exogenous SMase increases the levels of intracellular ceramide and subsequently induces apoptosis in human leukemia cell lines HL-60 and U937 [26]. In SK-N-MC cells, the addition of 400 mU/ml of exogenous bacterial SMase induced LDH leakage (data not shown) and internucleosomal DNA fragmentation (Fig. 1B).

3.2. The involvement of caspases in ceramide-induced apoptosis

Caspases have been shown to be implicated in various kinds of agent-induced apoptosis. To elucidate the involvement of caspases in C2-ceramide-induced apoptosis in SK-N-MC cells, we examined the effect of Z-Asp-CH_2-DCB, a caspase inhibitor, on C2-ceramide-induced internucleosomal DNA fragmentation. Pretreatment with Z-Asp-CH_2-DCB inhibited not only internucleosomal DNA fragmentation by C2-ceramide (Fig. 2A) but also exogenous SMase-mediated internucleosomal DNA fragmentation (data not shown) in a dose-dependent manner. Furthermore, we investigated whether PARP is cleaved by treatment with C2-ceramide, since PARP is a target of proteolytic cleavage by the caspases. Treatment with C2-ceramide, but not DHC2-ceramide, resulted in PARP cleavage, and the cleavage was inhibited by pretreatment with Z-Asp-CH_2-DCB in a dose-dependent manner (Fig. 2B). Subsequently, to investigate whether caspase activity increases in response to C2-ceramide, cytosolic extracts from C2-ceramide- or DHC2-ceramide-treated SK-N-MC cells were subjected to a protease activity assay using fluorogenic peptide substrates, DEVD-MCA and YVAD-MCA. Enzymatic cleavage of DEVD-MCA, which is specific to caspase-3-like proteases, slightly increased 3 h after the addition of C2-ceramide, and thereafter became elevated for up to 6 h in a caspase inhibitor-sensitive manner (Fig. 3A). On the other hand, we detected no enzymatic cleavage of

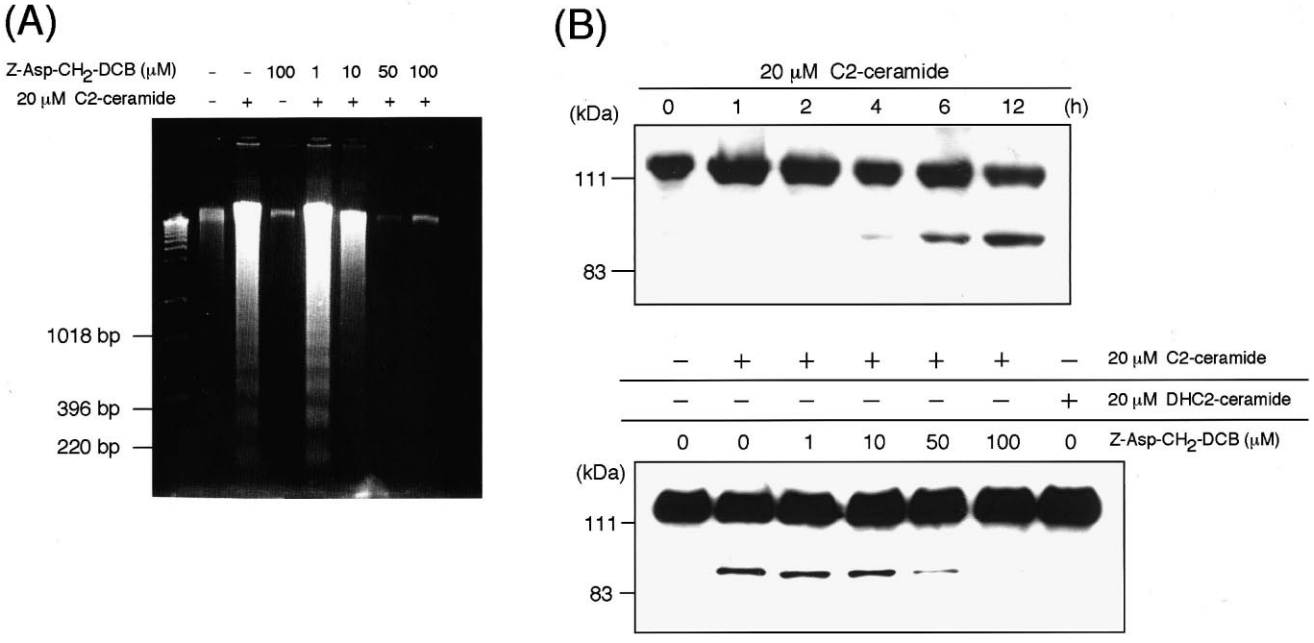

Fig. 2. Involvement of caspases in C2-ceramide-induced apoptosis in SK-N-MC cells. (A) Inhibition of C2-ceramide-induced DNA fragmentation by Z-Asp-CH_2-DCB, a non-selective caspase inhibitor. The cells were preincubated with various concentrations of Z-Asp-CH_2-DCB (0–100 μM) for 1 h and then incubated with 20 μM C2-ceramide for 6 h. Genomic DNAs were subjected to a DNA fragmentation assay. (B) Cleavage of PARP by treatment with C2-ceramide in SK-N-MC cells. The cells were pretreated with 100 μM Z-Asp-CH_2-DCB for 1 h before treatment with C2-ceramide for 6 h (B) or treated with 20 μM C2-ceramide for various periods (0–12 h) (A), and the membrane fractions (5 μg) were subjected to Western blot analysis using anti-PARP antibody.

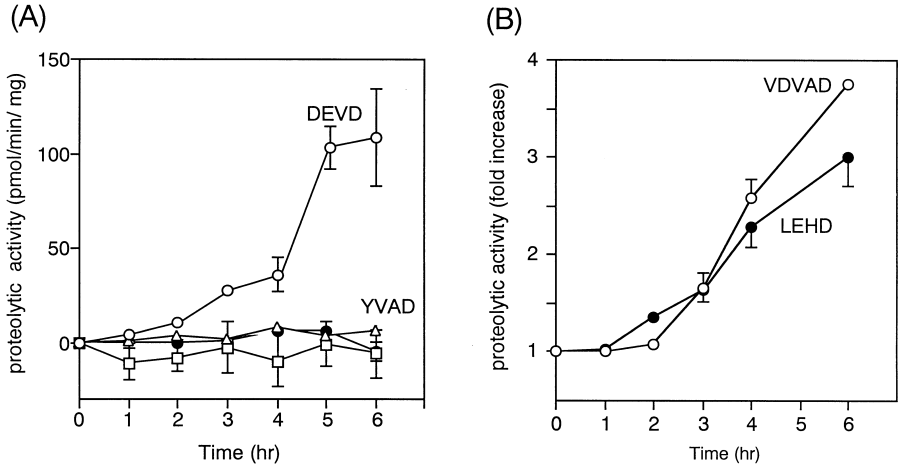

Fig. 3. Activation of caspases during apoptosis induced by C2-ceramide. (A) Activation of caspase-3-like, but not caspase-1-like proteases during C2-ceramide-induced apoptosis. The cells pretreated with or without Z-Asp-CH_2-DCB for 1 h were incubated with 20 μM C2-ceramide or 20 μM DHC2-ceramide for the various indicated periods. Released AMC from the fluorogenic substrates Ac-DEVD-AMC or Ac-YVAD-AMC were measured. ○, 20 μM C2-ceramide (DEVD); ●, 20 μM C2-ceramide+100 μM Z-Asp-CH_2-DCB (DEVD); △, 20 μM C2-ceramide (YVAD); □, 20 μM DHC2-ceramide (DEVD). Data are representative of three experiments. (B) Time course for caspase-2 and -9 activity in the cell extracts after C2-ceramide challenge. The cells were stimulated with 20 μM C2-ceramide for various periods. Then, cell lysates were prepared and an aliquot (200 μg) was used for caspase-2 or -9 assay. Comparison of the fluorescence of AFC from a sample with an unstimulated sample allows determination of the fold increase in caspase activity. Data are the mean of triplicate reactions run in parallel.

YVAD-MCA, which is specific to caspase-1-like protease activity, during C2-ceramide-induced apoptosis (Fig. 3A). Treatment with DHC2-ceramide did not induce the enzymatic cleavage of DEVD-MCA (Fig. 3A) or internucleosomal DNA fragmentation (Fig. 1B). We further examined VDVAD-AFC and LEHD-AFC cleaving activity, specific for caspase-2 and caspase-9, respectively. Enzymatic cleavage of VDVAD-AFC was significantly detected 3 h after being challenged with C2-ceramide, and was elevated with a time course similar to that of the cleavage of DEVD-MCA (Fig. 3B). LEHD-AFC cleavage was slightly detected as early as 2 h after treatment with C2-ceramide, and became thereafter elevated as seen in those of other substrates (Fig. 3B).

We next investigated which isoforms of caspases are expressed in SK-N-MC cells by RT-PCR methods. Total RNA was prepared from untreated and C2-ceramide-treated SK-N-MC cells and analyzed by qualitative RT-PCR (Fig. 4A). Caspases-2, -3, -4, -6, -7, -8, -9, and -10 but not caspases-1 and -5 were expressed in SK-N-MC cells. No significant differences in the expression levels of the eight caspase family members were observed between untreated and C2-ceramide-treated cells.

We then examined which isoforms of caspases were activated during apoptosis by C2-ceramide using antibodies specific to each caspase. Immunoblotting analysis revealed that proforms of caspases-2 and -3 decreased in C2-ceramide-treated cells in a time-dependent manner (Fig. 4B). On the other hand, caspases-6, -7, and -8 were detected in a quiescent state in SK-N-MC cells; however, the levels of these proteins did not change during apoptosis (Fig. 4B). The level of α-tubulin, used as an internal control, was not altered by treatment with C2-ceramide (Fig. 4B).

3.3. *The release of cytochrome c into the cytosol during ceramide-induced apoptosis*

Cytochrome *c*, which is released from mitochondria, has recently been identified as a key or important factor that can activate caspase-3 in a cell-free system [27]. In fact, it has been reported that levels of cytosolic cytochrome *c* are increased in response to various apoptotic stimuli such as staurosporine, etoposide, and ultraviolet-B irradiation [28,29]. We therefore tested whether levels of cytosolic cytochrome *c* increase in response to C2-ceramide. Cyto-

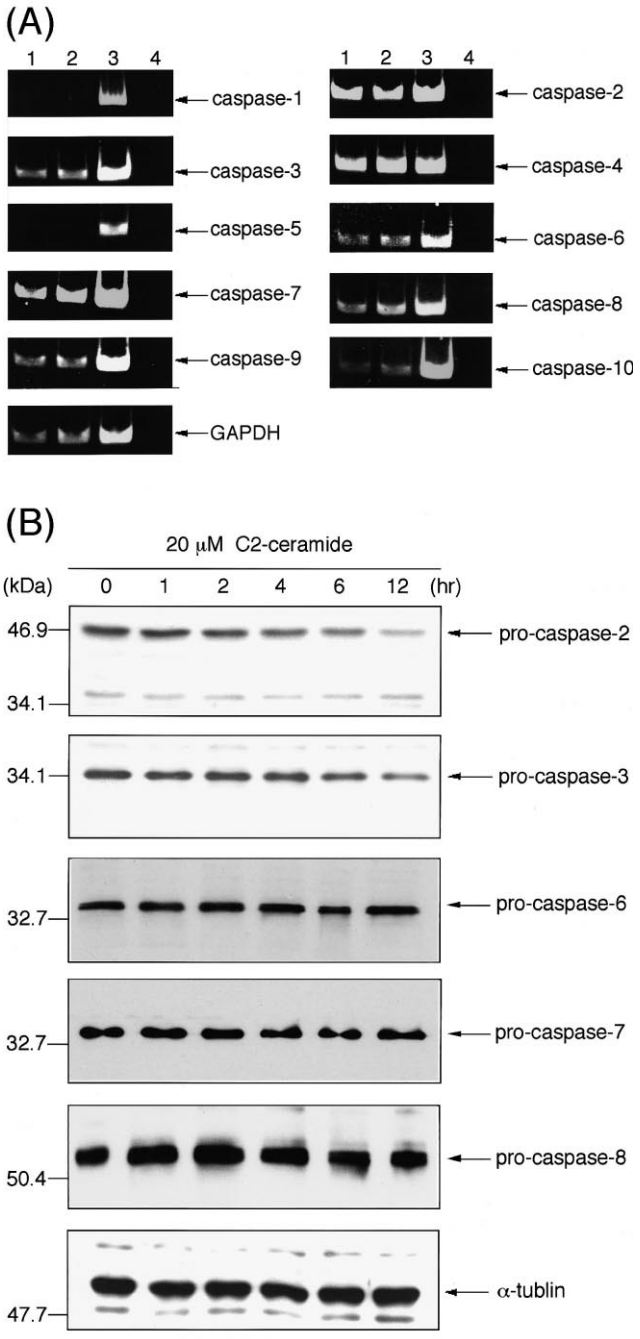

Fig. 4. Caspase mRNA expression and protein level during apoptosis. (A) Total RNA was isolated from ethanol vehicle (lane 1) and 20 μM C2-ceramide-treated (lane 2) SK-N-MC cells. RNA from Jurkat cells (lane 3) was used as a positive control. The analysis in lane 4 was carried out without a cDNA template as a negative control. Decrease of pro-caspase-2 and -3 in response to C2-ceramide in SK-N-MC cells. The cells were treated with 20 μM C2-ceramide for various periods (0–12 h). (B) The cytosol fractions were prepared, and an aliquot (20 μg) was then subjected to Western blot analysis using anti-ICH-1L (caspase-2), anti-CPP32 (caspase-3), anti-caspase-6, -7, and -8, and anti-α-tubulin antibodies.
←

chrome *c* in the cytosol fraction increased significantly after 2 h of being challenged with C2-ceramide, and this increase was detected for up to 12 h (Fig. 5). The time course of cytochrome *c* release into the cytosol fractions by treatment with C2-ceramide was coincident with that of enzymatic LEHD-AFC cleavage.

3.4. *Cytochrome c activated caspase-2 in cell-free system*

Although cytochrome *c* can activate caspase-9 in the presence of dATP and Apaf-1 (a human homologue of Ced-4) in a cell-free system [30], it is not known whether other caspases including caspase-2 can be activated by this cascade. We therefore examined the relationship between cytochrome *c* release and caspase-2 activation. The S-100 fraction of cytosol prepared from SK-N-MC cells contains a small amount of cytochrome *c* by homogenization (Fig. 6A). To remove the contaminated and leaked cytochrome *c*, a monoclonal antibody against cytochrome *c* was added into the S-100 fraction (designated S-100 (−cyt *c*)) (Fig. 6A). As shown in Fig.

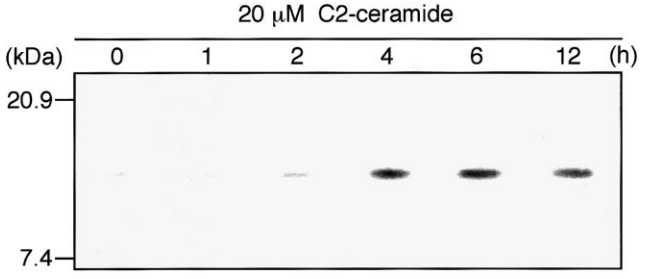

Fig. 5. Cytochrome *c* translocation to the cytoplasm induced by treatment with C2-ceramide. The cells were treated with 20 μM C2-ceramide for various periods (0–12 h), and the cytosol fractions (10 μg) were subjected to Western blot analysis using anti-cytochrome *c* antibody.

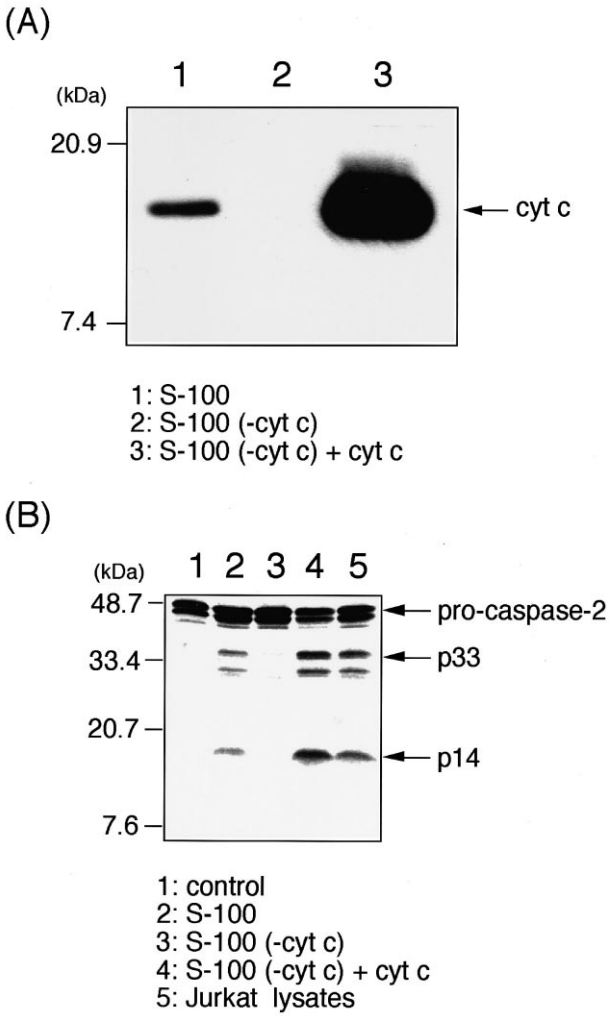

Fig. 6. In vitro reconstitution of caspase-2 activation reaction by cytochrome *c*. (A) Immunoblot analysis of cytochrome *c* from a sample (30 μg) of the S-100 fraction prepared from SK-N-MC cells (S-100) (lane 1), the S-100 fraction immunodepleted of cytochrome *c* (S-100 (−cyt *c*)) (lane 2), or S-100 (−cyt *c*) plus 0.2 μg of cytochrome *c* from horse heart (S-100 (−cyt *c*)+cyt *c*) (lane 3). (B) Samples (50 μg) of S-100, S-100 (−cyt *c*), or S-100 (−cyt *c*)+cyt *c* were incubated for 2 h with in vitro-translated ^{35}S-labeled caspase-2 in the presence of 1 mM dATP. Samples were subjected to 15% SDS-PAGE and analyzed by Fujix Bas 2000. Anti-Fas antibody-treated Jurkat cytosolic lysates (Jurkat lysates) were used as a positive control.

6B, the S-100 fraction could activate caspase-2 in the presence of dATP, as measured by the cleavage of pro-caspase-2. The S-100 (−cyt *c*) fraction lost the ability to activate caspase-2, and the activation was restored by the addition of purified cytochrome *c* (Fig. 6B).

4. Discussion

We have shown that C2-ceramide, a cell-permeable ceramide analogue, elicits apoptotic cell death as estimated by LDH leakage, internucleosomal DNA fragmentation, and chromatin condensation in SK-N-MC cells (Fig. 1). SMase, which hydrolyzes sphingomyelin to ceramide, also induced LDH leakage (data not shown) and DNA fragmentation (Fig. 1B), suggesting that the production of ceramide from sphingomyelin with the addition of SMase into the medium participates in the induction of apoptosis in SK-N-MC cells. We found that sphingosine, a metabolic product of ceramide, also induces several apoptotic features in this cell line along with caspase-3-like protease activation in a caspase inhibitor-sensitive manner (Fig. 1, data not shown), indicating that sphingosine has the ability to induce apoptosis as well as C2-ceramide in SK-N-MC cells. Therefore, it is likely that sphingosine functions as a second messenger in apoptosis. Alternatively, the intracellular production of sphingosine may contribute to apoptosis in response to ceramide. These results are supported by previous reports that C2-ceramide induces apoptosis in other cells, including neuronal cells [1–6]. In contrast, it has been reported that C2-ceramide protects against glutamate and amyloid β-peptide toxicity or trophic factor deprivation-mediated apoptosis [7,8], suggesting that the mechanism of signal transduction after ceramide production differs among the different types of neuronal cell lines. On the other hand, Hofmann and Dixit questioned the role of ceramide in apoptosis [31]. They question the existence of agonist-induced changes in sphingolipid levels and specifically challenge the use of the diglyceride kinase (DGK) assay for quantitation of ceramide, basing their argument on the results obtained by Watts et al. [32]. Hereafter, some papers objected to these assertions. Kolensnick and Hannun [33] mentioned the failure to include all pertinent data or misinterpretation of existing data and suggested that ceramide functions as a transducer of signals in a generalized stress-response pathway. They also reported that the appropriate use of DGK is critical for quantification of ceramide [34]. In response, Hofmann and Dixit [35], and Watts et al. [36] took objection to the comments. In view of these comments, the role of ceramide in apoptosis

remains controversial, because the quantification assay of ceramide is not yet fully established now. At least, some stresses such as heat shock, cytokine (including TNF-α), or hypoxia seem to increase the levels of ceramide during apoptosis. Therefore, we focus on the possible mechanism of apoptosis challenged with ceramide in this report.

Although the mechanism of ceramide-induced apoptosis is not clearly understood, it is thought that caspase-3 may be involved in apoptosis triggered by ceramide [16,21]. In SK-N-MC cells, pretreatment with a caspase inhibitor, Z-Asp-CH_2-DCB, significantly suppressed C2-ceramide-induced DNA fragmentation in a dose-dependent manner (Fig. 2A). Moreover, C2-ceramide, but not DHC2-ceramide, resulted in PARP cleavage, yielding an 85 kDa fragment showing typical caspase activity in a time- and caspase inhibitor-dependent manner (Fig. 2B). Subsequently, we tried to elucidate the subtypes of caspase which may be involved in ceramide-induced apoptosis. Caspase-3-like proteases, but not caspase-1-like proteases, were activated during apoptosis in response to C2-ceramide, and the activation was sensitive to Z-Asp-CH_2-DCB (Fig. 3A). On the other hand, DHC2-ceramide, a structural ceramide analogue with no biological effects, could not elicit either DNA fragmentation (Fig. 1B) or caspase-3-like protease activation (Fig. 3A). Furthermore, caspase-2 activity (VDVAD-AFC cleaving activity) was significantly detected with a time course as seen in that of caspase-3-like proteases activation (Fig. 3B). Although caspase-9 activity (LEHD-AFC cleaving activity) also increased in response to C2-ceramide, the activity was detected as early as 2 h after treatment (Fig. 3B). These results suggest that caspases including caspases-9, -2, and caspase-3-like proteases are probably involved in C2-ceramide-induced apoptosis. Western blot analysis showed that the level of pro-caspase-3 is decreased (Fig. 4B). These data are consistent with results showing that caspase-3, but not caspase-1, plays a key factor in various kinds of stimulus-mediated apoptosis [16,17,20–22]. We next examined whether the level of pro-caspase-2 is really decreased by treatment with C2-ceramide corresponding to the results of enzymatic fluorescent substrate cleavage. Fig. 4B shows that the level of pro-caspase-2 is decreased in response to C2-ceramide, suggesting the possibility that caspase-2 is also activated during ceramide-induced apoptosis. Recent reports have indicated that caspase-2 is an effector in the pathway leading to apoptosis induced by a variety of apoptotic stimuli such as etoposide, γ-irradiation, nitric oxide, hypoxia or trophic factor withdrawal [15,37–39]. We showed that caspase-2 may be involved in apoptosis in response to ceramide in SK-N-MC cells. However, further study is required to clarify the role of caspase-2 in ceramide-induced apoptosis.

Cytochrome *c* has recently been identified as one factor that activates caspase-9 in collaboration with Apaf-1 (a human homologue of Ced-4) and dATP [27,30,40]. In fact, the release of mitochondrial cytochrome *c* into cytosol is induced by apoptosis-eliciting agents or the overexpression of Bax [28,29,37,38,41]. We therefore examined whether cytochrome *c* is released into the cytosol during ceramide-induced apoptosis in SK-N-MC cells. As shown in Fig. 5, cytochrome *c* was detected in the cytosol fractions 2 h after C2-ceramide treatment. The appearance of cytosolic cytochrome *c* in response to C2-ceramide precedes the decrease of caspase-2 and -3 proforms, and increase in DEVD-MCA and VDVAD-AFC cleaving activity. However, both cytochrome *c* release into cytosol fraction and caspase-9 activation (LEHD-AFC cleavage) were detected as early as 2 h after treatment. These results suggest that cytochrome *c* release and caspase-9 activation lead to caspase-2 and -3 activation. Therefore, to investigate whether caspase-2 acts downstream from cytochrome *c* release the same as caspase-3, we examined the roles of cytochrome *c* on caspase-2 activation using a cell-free system. The examination using S-100 fractions prepared from SK-N-MC cells indicated that the activation of caspase-2 was induced by the addition of cytochrome *c* (Fig. 6). This suggested that cytochrome *c* may be able to activate caspase-2 either directly or indirectly. Caspase-2 is thought to be probably activated by caspase-3-like activity [42]. Therefore, caspase-2 may be activated by other caspases including caspase-3 by treatment with C2-ceramide in SK-N-MC cells as seen in Fas- or staurosporine-induced apoptosis [42]. Although we showed evidence that the proform of caspase-2 is decreased by treatment with C2-ceramide (possibly in a cytochrome *c*-dependent manner), there remains the problem of how caspase-2 is acti-

vated by the treatment in neuronal cells. Wang et al. have shown that cytochrome *c* activates caspase-3 through the formation of the caspase-9/Apaf-1 complex, and the physical association of caspase-9 and Apaf-1 is mediated by the interaction of their respective caspase recruitment domains (CARDs) [40]. Several other caspases, including caspases-1, -2, -4, -8, and -10, also contain a CARD-like domain in their NH_2 termini. Moreover, it has been reported that caspases-4 and -8 can associate with Apaf-1 through their respective CARDs [43]. We have shown that caspase-9 is activated by C2-ceramide in SK-N-MC cells (Fig. 4B). Hence, it appears possible that (1) cytochrome *c* directly activates caspase-2 through the putative adaptor protein which has a CARDs in its NH_2 terminus, as seen in caspase-9 activation, or (2) the activation of caspase-9 by the formation of the caspase-9/Apaf-1 complex which sequentially cleaves pro-caspase-2 either directly or indirectly.

In conclusion, we have demonstrated that C2-ceramide, a cell-permeable ceramide analogue, induces apoptosis accompanying the release of cytochrome *c* into the cytosol and activation of caspases-2, -3, and -9 in SK-N-MC cells. Our data show that the activation of caspase-2 is triggered by the addition of cytochrome *c* in lysates of SK-N-MC cells. In view of these results, the release of cytochrome *c* into the cytosol and the sequential activation of caspases-9, -3, and -2 may be partly implicated in ceramide-induced apoptosis in SK-N-MC cells.

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